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# Spheroplasts of Borrelia burgdorferi

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# SPHEROPLASTS OF *BORRELIA BURGDORFERI*

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By

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## DEDICATION

To Dr. Richard D. Sagers of Brigham Young University who was responsible for introducing me to the magical, unseen world that can only be observed with the magnification and resolution provided by the optical systems of the modern compound microscope.

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# BACTERIAL PROTOPLASTS AND SPHEROPLASTS AS RESEARCH MODELS

A protoplast is that portion of the cell that consists of the cytoplasm and the surrounding plasma membrane. The term has been used to describe plant, fungal and bacterial cell entities from which the entire cell wall has been removed. A spheroplast is a cell with its cell wall disrupted and partially removed. Protoplasts are created from gram positive bacteria, because the cell wall is usually completely removed by cell wall degradation processes. As a general rule, spheroplasts are prepared from gram negative organisms, because the intimate association of peptidoglycan (mucopolysaccharide) with the cytoplasmic membrane often prevents complete removal of the cell wall. However, as in all phases of biology, there are no absolutes, and instances of protoplast formation from gram negative cells have been reported (34). In addition, different investigators call the entity thus formed either "protoplast" or "spheroplast" regardless of cell etiology or completeness of wall removal. This investigator has chosen to refer to them as they are named in the individual articles.

The very fact that the cell wall could be removed either completely or partially, leaving an osmotically sensitive cell that is capable of certain metabolic activity, has provided investigators with a superb model for research. Production and manipulation of these structures present scientists with tools for studying the physical, biochemical, immunological and genetic systems in the living cell and with ways of localizing many structures and metabolites.

The purpose of this review is to describe some of the previous studies that have taken advantage of bacterial protoplasts and spheroplasts as research models and to look at current and future applications of these methods.

The first indication of bacterial protoplast formation came when it was realized that lysozyme would lyse certain bacteria. Although lysozyme had been used previously during studies on bacteriophages, one of the first attempts to describe the structural changes associated with lysis came in 1949 when Welshimer and Robinow (35) set out to study the lysis of *Bacillus megaterium* in response to lysozyme purified from egg whites. The progress of cell lysis was correlated with the ability of the cell to combine with dye. Staining intensity decreased as lysis progressed, an indication that the molecules that combined with the dye were either lost from the cell due to lysis or were degraded by the enzyme. Welshimer also noted that the cell rigidity was lost and he was able to stretch the lysozyme-treated cell into threads and cause them to bend and fold.

Earlier workers (32) had shown that the cell wall of *Micrococcus lysodeikticus* contained a mucopolysaccharide that was degraded into reducing sugars by lysozyme. By 1950, Salton (28) demonstrated that a suspension of *M. lysodeikticus* cell wall material was cleared by lysozyme. Examination with the electron microscope showed the apparent dissolution of the cell wall. He concluded that the cell wall consisted of "the insoluble highly polymerized mucopolysaccharide substrate of lysozyme" and that the enzyme degraded the entire cell wall structure.

At about this same time, Kern, *et al.* (11) reported that lysozyme enlarged the cell of *M. lysodeikticus* , then ruptured and dispersed cell contents. In 1953 Weibull (32) proposed that it might be possible to control conditions of lysis in order to isolate the protoplast from the cell wall. His work showed that the bacterial protoplast is osmotically unstable and bursts unless protected by a hypertonic sucrose solution. When *B. megaterium* was subjected to lysozyme in a 0.1 to 0.2 M sucrose solution, the spherical structure of the organism was preserved. His observations were compatible with those of Kern and

Welshimer, notably those of the cells swelling and bursting when not protected by a hypertonic solution and the flexibility of the cell when the wall had been removed.

Both gram negative and gram positive bacteria contain the mucopolysaccharide lysozyme substrate, but their resistance to the enzyme varies. Gram positive organisms are degraded without any further treatment, but gram negative bacteria must be pretreated in some manner before the enzyme becomes effective. Treatments include heat, solvent extraction, alkali, detergent and versene (EDTA) (21). In 1958 Repaske (27) reported on the action of TRIS and EDTA in bringing about lysis in certain gram negative organisms. He also noted the increased lytic activity that occurred as the pH was increased. In a 1958 monograph, Salton (29) came to the conclusion that gram negative bacteria have the same basic mucopolysaccharide structure as gram positive bacteria. The structural framework was said to consist of glucosamine, muramic acid, and certain amino acids, mainly alanine, glutamic acid and diaminopimelic acid (DAP). In addition a lipoprotein component was detected. Salton was able to reach these conclusions in part because of earlier studies on protoplast formation. Protoplasts of *Eschersichia coli* were formed by lysozyme only at high pH values or with versene or sodium dodecyl sulphate (SDS), both of which aggregate lipoprotein complexes. Protoplasts were also formed when the cells were exposed to media containing penicillin. Protoplast formation of *Vibrio metchnikovi* and *Salmonella gallinarum* in penicillin was accompanied by a loss of cell wall amino sugar (mucopolysaccharide) and DAP.

The production of bacterial protoplasts was concurrent with research that led to the elucidation of the structure and components of the bacterial cell wall. This was the beginning of the exploitation of this remarkable cell entity as a model for physical and biochemical research on the living cell.

Upon removal of all or part of the bacterial cell wall vast areas of cytoplasmic membrane are exposed. The result provides an ideal model for research into the mechanisms of membrane dynamics. As early as 1959, Few, *et al.* (7) were making use of the protoplasts of *M. lysodeikticus* to characterize surface ionogenic groups by means of micro-electrophoresis techniques. From data gained by pH-mobility relationships and use of thorium and uranyl ions to observe changes in surface charge densities, they concluded that the electrophoretic behavior of the intact bacteria, the protoplast, and defatted membranes was controlled by carboxyl and amine groups and that the membrane lipids were phosphate. His data showed evidence of a protein-carbohydrate surface and an internal lipid component of the cytoplasmic membrane.

The Sixties' decade was the heyday period of membrane analysis and use of protoplasts and spheroplasts as a research technique in that analysis. In 1967 Op Den Kamp, *et al.* (25) used protoplasts of *B. megaterium* to study the different compositions of the phospholipids that result from different glucose concentrations and pH levels in the bacterial cell growth media. Eisenberg and Corner (6) enumerated changes in pore size and stretching that occur in protoplasts under different osmotic conditions of the external environment. Temperature, hydrogen ion concentration, and size and shape of the solute molecules affect osmotic behavior. The authors found that changes in growth temperature led to changes in the lipid composition of the membrane, with higher growth temperatures resulting in increased production of saturated, unbranched fatty acids.

Corner and his associates (4) used protoplasts of *B. megaterium* to monitor the effects of osmotic solutes on the cell membrane. They found that the bacterial cell membrane acted as a differential dialysis membrane and could discriminate between different solutes. Furthermore, stretching of the membrane altered its permeability. The pore size of the stretched membrane increased, allowing larger solute molecules to enter the cell. Solute

influx was accompanied by an influx of water molecules, causing in turn more stretching and pore enlargement to allow influx of even larger solutes. The protoplast was capable of stretching to an enormous size before undergoing brittle fracture and bursting. This behavior was evidence of protein being a structural component of the membrane. The effects on protoplast swelling that applications of the protein cross-linker, formaldehyde, and the denaturing effects of urea led to the conclusion that protein is an important membrane structural component (6). Corner also cited studies by other workers reporting electron micrographic evidence of a protein framework in lipid-extracted protoplast membranes.

Scherrer, *et al.* (30) used a similar system to examine differences in molecular sieving between the cell wall and the cytoplasmic membrane. They studied the differential uptake of 50 different hydrophilic probing molecules and their effect on osmotic stability. Important insight was gained into the variation in pore sizes in the cell wall and cytoplasmic membrane as well as the different kinds of molecules the two structures were capable of accepting.

The protoplasts of *Streptococcus faecalis* were titrated with acid and alkali to study the ion-exchange properties of the bacterial cell (15). It was found that acidified protoplasts were more resistant to osmotic shock than their neutral counterparts. The reason for this behavior was unclear. It was postulated that it could be because of acid-induced changes in the membrane or possibly reduced internal osmolality due to of solute loss. The most important information from this study related to the positions of certain ions. It was found that potassium ions were free to move in and out of the cell membrane and act as an ionic counterbalance while the magnesium ions generally remained firmly attached to the membrane, protecting it against alkaline lysis.

The research on the cytoplasmic membrane and cell wall advanced our understanding of the morphology and composition of the gram negative cell envelope and gram positive cell wall. Observations of the process of protoplast and spheroplast formation provided clues to apparent differences between these two major groups of bacteria. During the lysozyme degradation of cell wall accompanying protoplast formation from gram positive organisms, an extrusion of mesosomes occurred (33). Gram negative bacteria have a simple cytoplasmic membrane with no such structures.

The content and location of phospholipid, lipopolysaccharide, mucopolysaccharide and protein components of the cell wall and outer membranes vary considerably between gram negative and gram positive bacteria. By 1972, Osborn, *et al.* (26) confirmed and correlated results of numerous previous studies concerning gram negative cell membrane composition. Sucrose density gradient centrifugation of the membranes remaining after spheroplast formation separated the different membrane types present in the gram negative organism. By creating spheroplasts from a *Salmonella typhimurium* mutant that incorporates [$^{14}$C] galactose into lipopolysaccharides, the ratio of lipopolysaccharide in the cytoplasmic and outer membrane fractions could be calculated. Over 80% of the lipopolysaccharide was found in the lower density outer membrane, while the cytoplasmic membrane contained higher amounts of phospholipids. The phospholipid composition of the two membranes varied significantly. The protein composition also varied, with the larger proteins appearing in the cytoplasmic membrane fraction.

After the rigid cell wall has been either removed or broken down, leaving an osmotically sensitive structure, the separation of the remaining components is comparatively simple. The protoplasts or spheroplasts can be broken up by more gentle means to avoid damaging structures as occurs with the harsher methods necessary to break up the intact cell wall. Nowhere was this concept illustrated more vividly than in the

alkaline phosphatase purification process described by Malamy and Horecker in 1964 (14). Earlier purification procedures started with cell fragments obtained either from treatment with the French press or from acetone extraction. Enzyme preparations from these methods were heavily contaminated with other proteins, and purification processes either altered the properties of the alkaline phosphatase or introduced other interfering substances. A major contaminant was phosphodiesterase, which prevented the isolated alkaline phosphatase from being used to study nucleotide sequences. The supernatant collected during the conversion of *E. coli* to spheroplasts contained alkaline phosphatase of high specific activity that could be crystallized and that retained virtually no contaminants.

In a series of related papers (18,19 & 20), Neu and Hepel reported on modifications of the Malamy-Horecker procedure that resulted in the release of several more viable enzymes. They included an acid phosphatase, cyclic phosphodiesterase, and a ribonuclease. These degradative enzymes were thought to be located at or near the cell surface.

Polyribosomes can be isolated by breaking up cells by a number of methods. One of these methods involves the preparation of spheroplasts from *E. coli* by means of penicillin application. Dresden and Hoagland (5) isolated a polyribosome-rich extract by modifying the lysozyme-induced spheroplast preparation procedure to prevent the lysozyme and EDTA from acting on the cell at the same time. These workers also noted that when spheroplasts were incubated in growth medium, polyribosomes were produced from apparently newly synthesized mRNA.

From the beginning, protoplasts, spheroplasts, and L-forms (wall-less forms capable of propagation) were targeted as models for studying the mechanisms of genetic transfer. As early as 1957, Hagiwara (10) at Osaka University and Chargoff, *et al.* (2) at Columbia University were attempting the transfer of deoxypentose nucleic acid both to and from protoplasts of *E.coli*. Some transformation appeared to occur and Hagiwari was able to

confirm that F+ strain transfer of genetic material to F- strains is a one-way process even at the subcellular level.

By 1960 protoplasts had been prepared to study the infection by viral DNA. Meyer *et al.* (16) and Guthrie and Sinsheiner (9) had moderate success when infecting protoplasts of *E.coli* with bacteriophage DNA. Guthrie and Sinsheiner concluded that infection depended on protoplast preparation procedures and ionic concentrations of the reagents. Meyer's group found that treatment of the isolated phage DNA affected infectivity of the protoplasts. DNase treatment of the phage DNA destroyed infectivity, whereas trypsin degradation of the protein had no effect, suggesting DNA as the active infective agent.

In a review published in 1976 Young, Birdsell and Bettinger (36) set forth the argument that the cell wall played an important role in competence. It appeared that the wall provides a barrier to DNA uptake that is partly released by the activity of cell wall-degrading enzymes. Indeed, certain binding sites appeared to be located on the cytoplasmic membrane. In most cases protoplast formation caused the loss of DNA binding sites. In this review three major avenues of genetic analysis utilizing protoplasts and L-forms were suggested. In the first of these avenues, DNA preparation, isolation of macromolecular DNA from protoplasts results in minimum physical and enzymatic damage, and the DNA thus isolated are efficient in DNA-mediated transformation. In the second avenue, genetic defects that cause L-forms could be studied, and finally rapid lysis of protoplasts following viral adsorption could be investigated to understand the processing of phage DNA. Other investigators (8, 31) considered genetic recombination by fusion of protoplasts in the presence of polyethylene glycol (PEG).

Because it is easy to transform, the exploitation of *E. coli* for the cloning and expression of genetic material from eucaryotic and other procaryotic sources has been a major research emphasis for some years. Many plasmids and bacteriophages that are

suitable *E. coli* vectors and numerous useful techniques for their manipulation have been developed. However, *E. coli* is not always an acceptable host for genetic material obtained from other bacteria. Some foreign gene products are selected against, and others are lethal. The biochemical pathways necessary for the expression of transformed gene products do not always occur in *E.coli.* (13).

In the majority of bacteria other than *E. coli,* useful techniques for efficient transfer of genetic material from one strain to another have not been fully developed. Protoplast transformation and fusion are useful techniques for effecting genetic recombination and transformation in many bacteria, especially those species important in agriculture and industry. The main strategy involves the PEG-induced uptake of DNA (24). It produces a high yield of plasmid-containing transformants and can induce uptake of linear and non-supercoiled circular plasmid DNA as well as the supercoiled form. Uptake efficiency decreases as the size of the DNA fragments increases. A second, related technique involves the PEG-mediated fusion of two protoplasts and the plasmid transfer or genomic recombination that results (8, 31).

Two major steps in any process that involves protoplast-mediated transformation are protoplast formation and subsequent regeneration of the cell wall. Optimization of conditions leading to successful protoplast transformation and regeneration has been the objective of numerous studies. In 1973 Okanishi, *et al.* (23) initiated research into the cultural conditions necessary for preparing protoplasts from species of *Streptomyces* and for the subsequent reversion to the filamentous state. The concentration of magnesium and calcium ions was found to be an important factor in both protoplast stabilization and reversion.

*Bacillus subtilis* has been the subject of much of the research concerning protoplast-mediated transformation. In 1978 Gabor and Hotchkiss (8) saw a need to improve and

stabilize the recombination and regeneration yields resulting from the fusion of *B. subtilis* protoplasts in the presence of PEG. The objective of such a fusion is the division of the two cell chromosomes and the subsequent recombination of genetic material. They found that the timing of the return of the fused protoplasts to a cell wall restoration medium was crucial. If cell wall restoration and division took place too soon, recombination did not occur. The use of an intermediate minimal medium was suggested.

In the past several years, techniques and conditions for successful protoplast formation and regeneration have been described for *Streptococcus lacis* (22, 12), *Clostridium pasturianum* (17), *Lactobacillis casei* (13), *L. plantarum* (1), *Streptococcus faecalis* (31) and two rumen anaerobes, *Fusobacterium varium* and *Enterococcus faecium* (3). The conditions for efficient protoplast formation and reversion have varied considerably between the different species and even between strains. For instance, the inclusion of gelatin, bovine serum albumin (BSA), and bovine serum in the regeneration medium has usually been necessary, but the concentrations required have differed.

In 1989 Badi and his associates (1) compared spheroplast and electroporation-mediated transformation of *L. plantarum*. Electroporation worked well for some strains, whereas spheroplast transformation was preferable for others. It appears that the use of spheroplasts and protoplasts will continue to be suitable intermediates in the transfer of genetic material from one microorganism to another.

For almost forty years, protoplasts and spheroplasts have served as models for researchers looking for answers to questions concerning the structure, biochemistry and genetics of the procaryotes. The role has changed from a model for the study of cell components to a mediator for improvement of strains. It appears these remarkable entities will continue to find a place in the continuing quest for information and manipulation of the microbial world.

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ABSTRACT

SPHEROPLASTS OF *BORRELIA BURGDORFERI*

by Mary Leigh Talbot

This thesis examines spheroplasts prepared from the spirochete, *Borrelia burgdorferi,* by treatment with lysozyme. It outlines optimum preparation conditions, examines the ultrastructure of the spheroplast, and evaluates certain metabolic capabilities. The spheroplasts prepared as outlined in this thesis lacked a cell wall but retained the cytoplasmic membrane and periplasmic flagella. They expanded and burst unless stabilized in hypertonic solution. Electron microscopic observations revealed portions of the outer membranes that were sloughed off the cells during this process and reformed as spherical vesicles. The periplasmic flagella were released from the confines of the outer membrane after a minimal washing procedure. Spheroplasts were capable of responding to elevated temperatures by synthesizing heat shock proteins, but they were unable to reproduce.

# INTRODUCTION

The bacterium, *Borrelia burgdorferi*, is a member of the order, *Spirochaetales*, and the family *Spirochaetaceae* . All of the species in the genus, *Borrelia* , are transmitted to vertebrates by hematophagous arthropods (2). *B. burgdorferi*, the causative agent of lyme disease, is transmitted to humans by the bite of the Ixodes tick, *Ixodes dammini* , in the eastern United States and *I. pacificus* in the western U.S. (7).

Lyme disease is a severe human illness exhibiting a broad range of clinical manifestations that, when left untreated, appear in three successive stages. In stage I, there is often a local rash, erythema chronicum migrans (ECM), at the site of the tick bite. This is followed by headaches, fatigue, low-grade fever, regional lymphadenopathy, and other flu-like symptoms. Appearing weeks to months later, stage II is characterized by heart involvement and meningitis. Stage III, which can follow weeks to years later, includes arthritis and neuropathies (8, 24). Because of the confusing array of symptoms that make lyme disease indistinguishable from numerous other diseases, including aseptic meningitis, multiple sclerosis, rheumatoid arthritis, and juvenile rheumatoid arthritis, serological identification of the disease is important (20). Some of the immunologically important proteins of *B. burgdorferi* are the 41 kDa flagellin, the 31 and 34 kDa outer surface proteins (Osp), A and B, and the 60 kDa common antigen (6,11). An early and prolonged immune response to the 41 kDa flagellin is characteristic of the disease (12).

Research is being carried out to determine the importance of the heat-shock proteins (HSP) produced by the organism when exposed to the mildly elevated temperatures encountered when going from the arthropod to its warm-blooded host (8, 9). In an effort to localize and isolate the HSPs, it was proposed to separate the cell membranes from the cytoplasm by first creating spheroplasts (Saxena, personal communication.).

Spirochetes had not previously been the subject of spheroplast formation; therefore, a study was undertaken to identify optimum preparation conditions, evaluate certain metabolic capabilities, and observe their ultrastructure. Observations indicated that increased levels of the heat shock protein, HSP I, are synthesized by the spheroplast in response to elevated temperature. The cytoplasmic membrane of the spheroplast appeared to remain intact and the periplasmic fibrils, or flagella, were readily released from the confines of the outer membrane (OM). Much of the outer surface proteins, Osp A and B, remained with the spheroplast; however, portions of the OM were sloughed off and reformed as spherical vesicles. The cell wall was not apparent in the spheroplast.

Spheroplasts and protoplasts are important in the development of systems involving the efficient transfer of DNA. A necessary adjunct to this process is the reversion of the transformed spheroplast to a normal reproductive state. Preliminary studies herein to evaluate the conditions necessary to cause the spheroplasts to revert were inconclusive.

## MATERIALS AND METHODS

**Bacterial Strain.** *Borrelia burgdorferi* B31 (Type strain, ATCC 35210) was grown at 34°C in BSK II medium (1), except that bovine serum albumin was reduced to 1% (wt/vol).

**Spheroplast preparation.** Spheroplasts were prepared by the methods of Birdsell and Cota-Robles (5) and Osborn, *et al.* (22). Briefly, cultures of *B. burgdorferi* were grown at 34°C to a concentration of not more than $5 \times 10^7$ cells/mL and harvested by centrifugation (1400 x g for 20 min at 22°C). The pellet was resuspended to its original volume with 0.01 M Tris buffer pH 7.9 (TB). After centrifugation (1400 x g for 10 min) the pellet was suspended to half the original volume with 0.01 M Tris buffer containing 0.5 M sucrose pH 7.8 (TSB). A stock lysozyme solution (Sigma Chemical Co., St. Louis, Mo.) containing 2.00 mg lysozyme/mL in TSB was prepared and stored at -80°C. Stock lysozyme was added to the cell suspension to achieve a final concentration of 20 µg/mL of the original *B. burgdorferi* culture. After a 10 min incubation at room temperature, the cell suspension was returned to the original volume by slowly underlaying 1.0 mM disodium ethylenediaminetetraacetate (EDTA), leaving the cells in a final solution of 0.01 M Tris buffer containing O.25 M sucrose, lysozyme prepared as above, and 1.0 mM EDTA (TSLE). The resulting spheroplasts were harvested by centrifugation (1400 x g for 10 min).

**Spheroplast culture.** Spheroplasts to be cultured were immediately placed in a modified BSK II medium without gelatin containing 1.0 mg/mL ampicillin and 3.5 % sucrose (wt/vol) and incubated at 34°C. Spheroplasts for regeneration studies were placed in BSK II medium.

**Observations and measurements.** Darkfield microscopic observations were made on wet mounts under a Zeiss Photomicroscope II. Cell counts were made under darkfield optics with a Petroff-Hausser counting chamber.

**Heat shock.** Spirochetes were maintained at 34°C at a density of $5 \times 10^7$ cells/ mL, harvested by centrifugation (1400 x g for 20 min) and resuspended to a concentration of $5 \times 10^8$ cells/mL in labeling medium (BSK II containing methionine deficient RPMI supplemented with L-glutamine, L-leucine and L-lysine (Sigma Chemical Co.).

[$^{35}$S]methionine (ICN Pharmaceuticals Inc., Irvine, CA) was added to achieve a final concentration of 200 μCi/mL; then 1 mL aliquots containing spheroplasts (freshly prepared as above) or spirochetes were incubated at 30° or 38°C for 4 h and harvested by centrifugation (1400 x g for 10 min). Spirochetes were washed 3 times in phosphate buffered saline (PBS), and the spheroplasts washed 3 times in 5% sucrose before resuspension in 100 μL 0.5 M Tris hydrochloride, pH 6.8.

**Analysis of protein synthesis by SDS-PAGE, fluorography and scanning laser densitometry.** The protein concentration of each sample was determined by the method of Lowry, *et al.* (18), and counts per minute (CPM) were determined by liquid scintillation (Packard Instrument Co, Meriden, Ct.). For sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE), the samples were suspended in sample buffer with 2-mercaptoethanol (5% wt/vol) and heated for 1 minute at 100°C. The labeled spirochetes and spheroplasts (100 μg protein per lane) were applied to SDS-PAGE gels under reducing conditions by the method of Laemmmli and Favre (16). Samples were electrophoresed at 30 mA for 4 h under reducing conditions using a 5% acrylamide stacking and a 10% running gel. The gels were stained with Coomassie brilliant blue, enhanced for fluorography with Fluoro-hance$^{tm}$ (RPI) autoradiography enhancer, and exposed to photographic film (XAR-5; Eastman Kodak Co., Rochester, N.Y) at -80°C.

The film was developed and quantified with a scanning laser densitometer (UltraScan XL; LKB Instruments, Bromma, Sweden.)

**Specimens for electron microscopy.** Because spheroplasts have a structure analogous to that of mycoplasmas, we adapted procedures used to fix and embed mycoplasmas in preparation for thin sectioning (10). Briefly, the pellet was washed once in a modified BSK II medium containing 6% sucrose in order to stabilize the spheroplasts and remove the TB. The pellet was suspended in 2.5% glutaraldehyde (Sigma Chemical Co.) in modified BSK II medium and fixed for 2 h at 4°C. After washing with 0.05 M Na-cacodylate buffer pH 7.4, the suspension was embedded in agar blocks and fixed in 2% osmium tetroxide. The cubes were post-fixed in 2% aqueous uranyl acetate and dehydrated with ethanol. The agar blocks were infiltrated then embedded in Spurr's medium (27).

Thin sections were stained with 2% aqueous uranyl acetate and Reynold's lead citrate (23), cut with a glass knife, and observed with a Zeiss EM109 or a JEOL100S electron microscope at 50 or 80 kV. Photographs were taken with Kodak Technical Pan 6415 film or Kodak electron image film SO-163.

Negative staining was done on *B. burgdorferi* before and after each stage of spheroplast preparation. The spheroplasts were washed once in a PBS solution containing 5% sucrose, then suspended in PBS. The suspension was immediately stained with 2% phosphotungstic acid in equal volume with 100 µg/mL bacitracin and observed as above.

**Spheroplast fractionation.** The spheroplasts were repeatedly disrupted by forcing them through a 27 gauge needle. The supernatant from this preparation was concentrated by lyophilization and analyzed by SDS-PAGE.

**Replication studies.** For replication studies limiting dilutions were make to effect cell counts of 0-1 x $10^6$ cells/mL.

**Regeneration studies.** Under darkfield optics, it was impossible to distinguish partial spheroplasts with minimal cell wall damage from spirochetes that were unaffected by the lysozyme treatment. Consequently it was not possible to determine whether an increase in numbers after lysozyme treatment was due to multiplication of regenerated spheroplasts or unaffected spirochetes. For this reason any destruction of the peptidoglycan (PG) layer of the cell wall was taken as a measure of osmotic sensitivity. When a culture was treated with a hypotonic solution, only osmotically resistant cells would be able to grow. When dilutions were made in isotonic media (BSK II), both osmotically resistant and regenerated osmotically sensitive cells would grow. Differences in numbers of surviving cells under these two conditions could be used to calculate a regeneration frequency [ rf = (# of pos wells under non-lysing conditions - # of pos under lysing conditions/initial cell number )X 100 ].

The ability of spheroplasts to regenerate into spirochetes was evaluated by a modification of the method described by Lee-Wickner and Chassy for the regeneration of *Lactobacillus casei* protoplasts (17). *Borrelia burgdorferi* was grown to a concentration of $4.5 \times 10^7$ cells/mL in BSK II, centrifuged (1400 x g for 20 min), and resuspended to half the original volume in TSB. The culture was divided into two aliquots. Each aliquot was incubated with stock lysozyme to achieve a final concentration of 20 µg/mL of the original *B. burgdorferi* culture. After a 30 second incubation, the aliquot to be incubated under lysing conditions was diluted 1:10 in 2.0% sucrose, and the aliquot under non-lysing conditions was diluted 1:10 in BSK II. Each was enriched with 20 µg/mL N-acetylglucosamine. After a 3.0 min incubation, ten-fold dilutions in BSK II were made to effect cell counts of *ca.* $1 \times 10^6$ to 10 cells/mL. Each dilution (100 µL) was placed in each of 12 wells (one row) of a 96-well microtiter plate. After incubation at 37°C in a 5% $CO_2$-

enriched environment for 10 days, material from the wells was observed under darkfield optics for the presence of spirochetes.

## RESULTS

**Production of spheroplasts.** Spirochetes (90-95%) of *B. burgdorferi* were readily converted to stable spheroplasts at room temperature. The conditions provided by TB apparently weakened the outer membrane, allowing the lysozyme access to the PG layer. The 0.5 M TSB caused plasmolysis of the cell and a subsequent pulling away of the cytoplasmic membrane from the cell wall (5). As the sucrose solution was diluted to 0.25 M with EDTA, the cell wall was further disrupted and the plasmolyzed cell took up fluid and assumed a spherical shape. Lysozyme concentration, activity, and lot numbers were not critical, but several other factors were. The pH of the reagents, especially the pH of the final TSLE solution, was important (Fig.1). When the pH of the TB was above 8.0, conversion rates increased; however, the resulting spheroplasts were fragile and came apart easily. The number of spirochetes/mL in the starting culture was also important. If the count was greater than $5 \times 10^7$ cell/mL, the percentage converted to spheroplasts dropped . If the starting numbers were low, $2 \times 10^7$ cells/mL or less, the conversion rate was very high, but the resulting spheroplasts were fragile and easily broken.

Darkfield microscopic observation revealed spherical, semi-translucent cells having a diameter of about 2 μm. The freshly prepared spheroplasts had a tendency to aggregate in large clumps while in the TSLE upon completion of the procedure. They could also be maintained in an osmotically stable condition in 5% (wt/vol) sucrose.

When freshly prepared spheroplasts were placed in modified BSK II medium, the clumps disintegrated and the individual cells were easily observed. The spheroplast culture contained, at various times, four morphological cell types: typical spheroplasts, partial spheroplasts, residual spirochetes, and atypical spheroplasts. (Fig. 2). The majority of the cells were typical spheroplasts. After the first day in the culture medium, one or two

"tufts" became evident, usually extending distally from the cell. The spheroplast sometimes had a single, long, slender filament extending from it. A partial spheroplast was present in fresh preparations, appearing as a spirochete with a ballooned section. This cell usually quickly disappeared from the culture. As the cultures aged, the typical spheroplasts became atypical. They became oblong in appearance before expiring. The proportion of the residual spirochetes gradually decreased (Fig. 3).

Although the total number of cells in the spheroplast culture dropped dramatically during the first few days, a stable population of spheroplasts remained, often for as long as 6-8 weeks (Fig. 3). There was no evidence of spheroplasts undergoing cell division in any of the cultures.

**Limiting dilutions**. Limiting dilutions were carried out to determine if the spheroplasts were capable of replication. Direct, accurate cell counts on freshly prepared spheroplasts were difficult to obtain because of clumping. Cells that had been dispersed by maintaining them in BSK II were used for this study. After eight weeks, tubes were observed under darkfield optics. No increase in number of spheroplasts was observed under the conditions studied.

**Protein analysis.** The spheroplasts were incubated at two temperatures, 30° and 38°C, in the presence of [$^{35}$S]methionine to detect proteins synthesized during thermoregulation. Spheroplasts incorporated 10% or less label than that taken up by the intact spirochete growing at the same temperature (measured in CPM/μL). Approximately 10% of the residual spirochetes that were not converted to spheroplasts could account for this activity. The increase in total protein synthesis by untreated spirochetes when transferred from 30° to 38°C was 8%. The increase in percent labelled protein experienced by spheroplasts under these same conditions was 65% .

Proteins newly synthesized, as indicated by incorporated label, occurred across the entire molecular weight spectrum (Fig. 4). Flagellin represents a major protein band in both spirochetes and spheroplasts, and its increase as a percent of total protein from 30° to 38°C was about 4.3% for spirochetes and slightly less for spheroplasts (Table 1). The flagellin band in spheroplasts was also of a slightly lower molecular weight than that of the intact spirochetes (Figs. 4, 5).

The total number of protein bands showing newly incorporated label was greater in the spheroplast samples; however, the size of most bands represented a smaller percentage of the total protein (Table 1). The major exception to this trend was heat shock protein 1 (HSP 1). In the spirochetes, HSP I increased 2.3% when transferred from the lower to the higher temperature, and spheroplasts demonstrated a 3.9% increase in HSP I as a percentage of total protein (Table 1). The outer surface proteins, Osp A and B, increased slightly in spheroplasts in comparison to spirochetes.

The SDS-PAGE profiles of spirochetes and spheroplasts were qualitatively similar (Fig. 5). The Osp's located between 31 and 34 kDa are of particular importance. The Osp A and Osp B bands were evident in both the Coomassie-stained gels and the fluorograms. At least one band corresponding to 31.5 kDa was synthesized by intact spirochetes but not by spheroplasts (arrow, Fig 4).

**Ultrastructure.** In thin sections, spheroplasts had a diameter of *ca.* 0.5 μm (Fig. 6). They bore an extended outer membrane (OM) surrounding a cytoplasmic membrane, but PG was not apparent. Spirochetes prepared by the same method appeared to have intact OM, PG, and the cytoplasmic membrane surrounded a much more electron-dense area (Fig.7). When viewed in transverse section, the diameter of the intact spirochete was about 0.2 μm. Numerous spherical membrane-bound vesicles were present in the vicinity of the spirochetes and spheroplasts (Fig. 6).

Negative staining preparations of intact spirochetes showed slender, coiled structures with intact OM and PG (Fig. 8). After the TB wash, the spirochetes remained unchanged; however, the coils lost their regularity and the OM was disrupted (Figs. 9, 10). In most of the TB-washed spirochetes periplasmic flagella had worked themselves loose from the confines of the OM (Figs. 9, 11, 12). After suspension in TSB and incubation in lysozyme, negatively stained spirochetes were generally cylindrical, but the cell boundary was disrupted in areas (Fig. 11.B).

PBS-suspended spheroplasts (which had been treated with TSLE) were as much as 3.0 μm in diameter and had developed ruptures in the membrane (Fig. 12). Both typical spheroplasts and partial spheroplasts (Fig. 13) were present, and structures resembling periplasmic flagella were evident on and near all of the spheroplasts (Fig. 12, 13).

**Spheroplast fractionation.** Darkfield microscopic examination of the spheroplasts indicated a tuft at either pole. Negative stains of spheroplasts also showed numerous long strands of periplasmic flagella that were released from the confines of the OM. Attempts to separate and identify the tufts by disrupting the spheroplasts, centrifuging them, and observing the supernatant by means of SDS-PAGE demonstrated minimal amounts of flagellin in the supernatant, and the preparation contained numerous other proteins (Fig. 14).

**Regeneration studies.** In one preliminary experiment in which spirochetes were subjected to lysing conditions, growth occurred in all wells of the microtiter plate that contained $10^2$ cells and in half of the wells containing *ca.* 10 cells. There was no growth in the wells that contained *ca.*1 cell/well. In the plate containing cells incubated in nonlysing conditions, there was growth in all the wells containing $10^2$ cells/well and in 11 of the 12 wells containing 10 cells/well. Five of the 12 wells containing *ca.* 1 cell/well had growth.

## DISCUSSION

The spirochete, *Borrelia burgdorferi,* is readily converted to spheroplasts by methods previously employed on the gram negative organisms, *E. coli* and *Salmonella typhimurium.* The cell envelope of a spirochete is analogous to that of other gram negative bacteria, having a trilaminar outer membrane (OM), an underlying periplasmic space, and a narrow peptidoglycan (PG) layer just external to the cytoplasmic membrane (2).

Normally the OM protects the PG from attack by degrading enzymes such as lysozyme; therefore, pre-treatment is necessary to allow the enzyme access to the underlying PG layer. The slightly alkaline pH of the TB was adequate for this purpose. If the pH of the TB or the combined pH of the TSLE dropped below 7.5, the percentage of spirochetes converted to spheroplasts dropped, probably because the pH was not sufficiently high to cause disruption of the OM prior to addition of the enzyme. When the pH was high (greater than 8.0 ) the spheroplasts were destroyed, presumably due to destruction of the underlying cytoplasmic membrane. Optimum pH conditions allowed the PG to be degraded but left the OM intact.

Bits of the OM appeared to be sloughed off and reformed into spherical vesicles. Surface antigens associated with extracellular membrane vesicles in *Borrelia burgdorferi* have been observed both *in vitro* and *in vivo* (D.W. Dorward, T.G. Schwan and C.F. Garon, Abstr. Annu. Meet. Am. Soc. Microbiol. 1991, C-310; 1990, D-131). Portions of the OM released as vesicles after phosphate buffer (PB) wash, as reported by Holt (15), could be seen both in thin sections and negative stains. In some of the negative stains portions of the OM were observed pulling away as spherical blebs. Evidently the OM was resistant to both chemical and mechanical disruption and could reform as a protective layer around the bacterium. Two proteins, the 31 kDa Osp A and the 34 kDa Osp B, which are

encoded on an extrachromasomal plasmid, are an integral part of the OM. Chronic infection is characterized by an immune response to these two proteins late in the disease (19). The relative amounts of Osp A and Osp B present in intact spirochetes and cultures that had undergone treatment for spheroplast conversion varied little, indicating that the OM remained relatively intact.

Careful inspection of the proteins in the 31 to 34 kDa range in SDS-PAGE preparations in this study and elsewhere indicated extensive variability (9, 19). Schwan, *et al.* (25) reported the virtual disappearance of Osp B expression upon continued passage in BSK II. The type strain used in current experiments had been passed numerous times. Because the immunological response to these two proteins occurs late in the course of lyme disease, it has been proposed that either the OM is damaged during tick to host transfer and requires a period of time to mend or that the two OM proteins are not expressed under certain conditions (11, 19, 6). Each of the Osp's represent only 1.5% of the total protein in the *B. burgdorferi* in this study, compared to 5 to 7% of the total protein in the bacterial strain in the study of Cluss and Boothby (9). Further research is called for to pass a low expression organism in an appropriate animal model to see if the expression of these proteins is affected.

Application of rRNA homology and oligionucleotide cataloging to members of the order, *Spirochaetales,* indicates it could occupy its own phylum (2, 3). Morphologically the major difference between spirochetes and other gram negative organisms is the former's containment of flagella within the periplasmic space. During the course of lyme disease, the specific IgM response is at a peak in the third to sixth week after the onset of illness, can persist for months to years, and is directed mainly against the 41 kDa protein, flagellin. The initial IgG response peaks some three weeks after IgM and is limited to the 41 kDa protein, expanding to other *Borrelia* proteins, including the OM proteins, during prolonged

infection (12). Careful observation of *B. burgdorferi* ultrastructure during spheroplast preparation can provide insight into this dichotomy. Periplasmic flagella were not observed in the thin sections but were evident in almost all of the negative stains. After only a PBS wash before fixation, the intact spirochete showed only a few flagella that had apparently worked free of the OM. As the spirochete underwent washings with TB, sucrose, and EDTA, the extruded flagella became more evident. Spheroplasts devoid of any visible OM or PG had clusters of flagella extending from them. Mechanical disruption to the spirochete upon transfer from the tick to the warm-blooded host and the attack on the organism by the host immune system could expose the flagella in a similar manner, thus making flagella an ideal target for immune responses.

At the molecular level and to some extent the morphological level, there are major differences among the flagella of the different spirochete genera. For instance, the outer sheath observed on treponeme flagella is not present in *Borrelia* (2). Monoclonal antibodies have been produced against the 41 kDa flagellin antigen in *Borrelia*, and an ELISA developed for diagnosis of syphilis employs flagella of *Treponema phagedenis* as test antigens (13). Recent studies indicate that species-specific epitopes are located in the middle of the flagellin molecule and can be exploited for species' identification (28). The individuality of flagellin combined with the strong and persistent antibody response makes it an ideal antigen for use in identification of the infection. Because of the abundance of extruded flagella in spheroplast preparations, an attempt was made to separate the flagella by mechanical disruption and centrifugation. The fragile cytoplasmic membrane was probably disrupted before the flagella could be completely removed. The flagellin was divided between fractions containing numerous proteins. Perhaps the cell should be disrupted after the wash with TB when the flagella are free but the PG is intact. A more

resilient cell would resist breaking up before the flagella could be removed. Further experimentation with centrifugation times is also suggested.

The PG layer of the cell wall of *Borrelia* has not been well characterized. It has been determined that the electron-dense layer just external to the cytoplasmic membrane is PG, as it contains muramic acid and the diamino acid, ornithine (2). In earlier studies on the same material in *Spirocheata stenostrepta*, penicillin treatment caused the protoplasmic cylinder to become distorted, and lysozyme treatment caused the disappearance of the electron-dense layer, both indications of a cell wall composed of PG (15). Ultrastructural observations of *B. burgdorferi* before lysozyme treatment showed an electron-dense layer surrounding the protoplasmic cylinder. After treatment with lysozyme and EDTA the electron dense layer disappeared leaving both the cytoplasmic and outer membranes clearly visible. The disappearance of this material was concomitant with the change in the helical shape of the spirochete to a sphere, implying that the rigid, structure-forming material is a lysozyme substrate.

The Osps, A and B, along with the 41 kDa flagellin of *B. burgdorferi*, have been cloned in *E. coli*, and the *E. coli* genetic system may be used for isolation and production of other proteins (4, 28). However, *E. coli* does not always express gene products of heterologous genes (17). Transformation of endogenous genes and/or modified genetic material between different *B. burgdorferi* strains could become an important research tool. Spheroplasts lacking an intact OM and cell wall may have increased competence useful in genetic manipulation and be capable of regenerating into intact, genetically altered but functional spirochetes.

Attacking the bacterial cell wall with lysozyme to create spheroplasts or protoplasts renders many bacteria competent, that is, able to effect a change in genetic material either by taking up DNA directly through the cell membrane or by fusing protoplasts (21, 26). For

either of these processes to be of value, the transformed bacteria need to be capable of regenerating the PG layer and subsequently replicating. For replication to occur, the spheroplast usually requires some PG to remain attached to the cell membrane to act as a primer for generation of the new cell wall (17). In the regeneration study described in this investigation, conditions were altered to minimize the amount of PG destroyed in order to maximize chances of wall regeneration and replication.

One preliminary study was inconclusive but interesting. In those dilutions representing approximately 10 cells/well, there was more growth in cells incubated under nonlysing conditions than in those incubated under lysing conditions. Further exploration of this method is warranted. For accurate calculation of regeneration frequencies, extensive replications of more dilutions must be evaluated. The spirochetes survived an initial exposure to TSB and lysozyme incubation. It was established that dilution and growth in microtiter wells could replace counting colony forming units (CFUs) on solid media. However, the necessity or absence of a preliminary TB wash, the amount and times of lysozyme incubations, and need the for additional N-acetyl glucosamine must be determined.

The definitions of spheroplasts and protoplasts do not take into account replication of the organism. The term, L-phase, is reserved for those variants of bacteria that lack cell walls, are an independent strain, and will replicate. Some enzyme-induced protoplasts have been found to replicate. They are L-phase variants that are obtained from enzyme-induced protoplasts (14). The spheroplasts of *B. burgdorferi* produced by the methods presented in this paper did not replicate. When placed in modified BSK II, the total number of spheroplasts increased initially but later were depleted in a continuous and irreversible manner. The increase can be attributed to the spheroplasts aggregating in large

clumps in the aqueous TSLE and slowly dissociating when placed in the more hydrophobic BSK II.

Thermoregulated protein synthesis in *B. burgdorferi* has been demonstrated (8, 9). Translational capabilities in response to changes in temperature were studied in spheroplast preparations to evaluate their ability to respond to a stimulus. Coomassie blue-stained preparations of SDS-PAGE gels of spirochetes and spheroplasts were similar. A 41 kDa molecule (flagellin) was altered and was lower in molecular weight as though the strands of flagellin may have been partially sheared during the process of making the spheroplasts. There was also an increase in protein at 66 kDa in the spheroplast lanes that was not present in the spirochete lanes. The spirochetes and spheroplasts were treated by the same method. Presumably the BSA present in BSK II was easily washed from the spirochetes but heavily contaminated spheroplast preparations.

Translational capabilities were determined by [$^{35}$S]methionine-labelled protein evident on fluorograms from SDS-PAGE of spirochetes and spheroplasts at 30° and 38°C. New protein synthesized by the spheroplasts could be due to the residual spirochetes. Spheroplasts incorporated only 10% of the label that the spirochete culture took in. However, when the increase in incorporated label was analyzed, with a temperature increase from 30 - 38°C, the spirochetes increased by 8%, whereas the spheroplasts increased by 65%. If the label incorporated by the spheroplasts was due only to the residual spirochetes, the increase should have been of the same magnitude as that in the spirochete culture. Perhaps the spirochetes were active at both temperatures and increased production of certain stress-induced proteins at 38°C. Conversely, the spheroplasts are not as metabolically active at 30°C, and at 38°C the incorporated label reflected both an increase in synthesis of regular proteins and stress-related proteins. Another consideration is that some or all of the organisms that appeared morphologically similar to residual

spirochetes under darkfield optics could have been PG damaged and still retained their helical shape. The amount of activity of the organisms with different degrees of PG destruction was not determined.

When the fluorogram was analyzed by laser densitometry, intact *B. burgdorferi* averaged 44 bands of newly synthesized protein while the spheroplasts had an average of 58 bands. Perhaps some subunits that are made in the cytoplasm are not assembled into complete proteins in the cytoplasm. Some enzymes needed for protein assembly in the perisplasmic space may have been lost or damaged during the spheroplast conversion process, resulting in more and smaller protein subunits. Some of the larger proteins might also have been disrupted during the conversion process. Flagellin, for example, could be sensitive to both of these processes. There is less flagellin synthesized by the spheroplast culture, the overall molecular weights are lower, and the flagellin is spread over a wider molecular weight range.

When organisms are exposed to elevated temperatures, they respond by repressing synthesis of some proteins in order to synthesize a small number of highly conserved heat shock proteins (HSP) (8). Several HSPs are produced by *B. burgdorferi* when the spirochete is transferred from 30° to 38°C. Heat shock protein I (73 kDa in this study), which is present in high amounts at 38°C, represented a greater proportion of total protein in the spheroplast than in spirochetes at both 30° and 38°C. This stress-related protein could increase because of both the temperature increase and the stress related to the lysozyme attack and associated washing procedures.

The spirochete, *B. burgdorferi*, was converted to a spheroplast with minimal apparent damage to the OM, the cytoplasmic membrane, and most major proteins. The spheroplasts demonstrated limited metabolic activity, as indicated by their ability to incorporate labelled methionine in the synthesis of new protein in response to changes in temperature. Analysis

of the proteins synthesized showed that the heat shock proteins are present in higher percentages in spheroplasts than in spirochetes. Spheroplasts have a highly extensible cytoplasmic membrane and must be stabilized in a hypertonic sucrose solution to prevent bursting in the absence of a cell wall. Careful inspection during the steps leading to spheroplast production indicated they may mimic stressed cells under attack by the host's immune system during infections. Minimal mechanical disruption of the spirochete caused the release of periplasmic flagella from the confines of the OM. This release may result in the early and prolonged immune response to flagellin observed during the course of lyme disease.

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TABLE 1. Quantification of protein synthesis in spirochetes and spheroplasts at 30 and 38°C*

| Cell type | Protein | ~ mol wt | % total protein at 30°C | % total protein at 38°C | per cent change from 30-38° C | change Bb-Sph at 38°C |
|---|---|---|---|---|---|---|
| *B. burgdorferi* | Flagellin | 39,000 | 15.9 | 20.2 | + 4.3 | -7.3 |
| Spheroplast | Flagellin | 38,000 | 14.0 | 12.9 | - 1.1 | |
| *B. burgdorferi* | HSP I | 73,000 | 2.0 | 4.3 | + 2.3 | +5.4 |
| Spheroplast | HSP I | 73,000 | 5.8 | 9.7 | + 3.9 | |
| *B. burgdorferi* | OSP-A | 32,000 | | 1.4 | | +1.0 |
| Spheroplast | OSP-A | 32,000 | | 2.4 | | |
| *B. burgdorferi* | OSP-B | 34,000 | | 1.5 | | +0.1 |
| Spheroplast | OSP-B | 34,000 | | 1.6 | | |

* As determined by scanning laser densitometry of Fig. 4.

Abbreviations: Bb, *B. burgdorferi*; Sph, spheroplast.

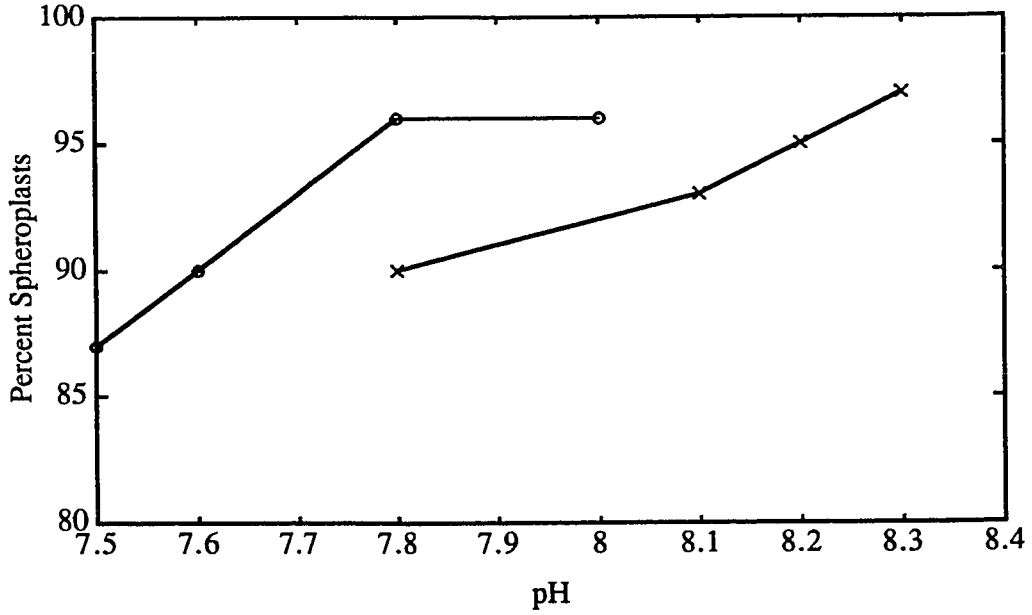


FIG. 1. Effect of the pH of the TB and TSLE on final percent conversion of spirochetes to spheroplasts. Symbols: o, TB: x, TSLE.

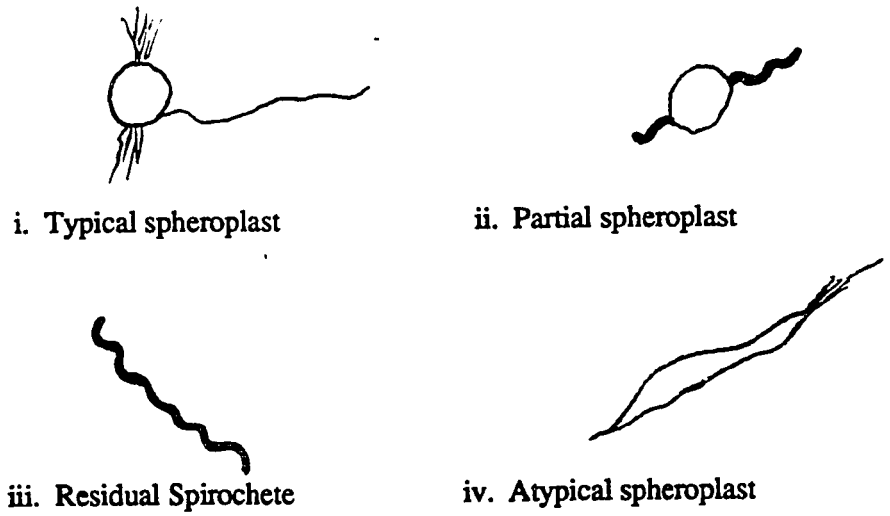


FIG. 2. Four cell types observed in spheroplast preparation. *i.* Typical spherplast with a tuft at either pole and often a long, fine thread extending from the cell. *ii.* A spirochete which has been partially converted to a spheroplast. *iii.* A spirochete unaffected by conversion process. *iv.* Atypical spheroplast often seen in older culture.

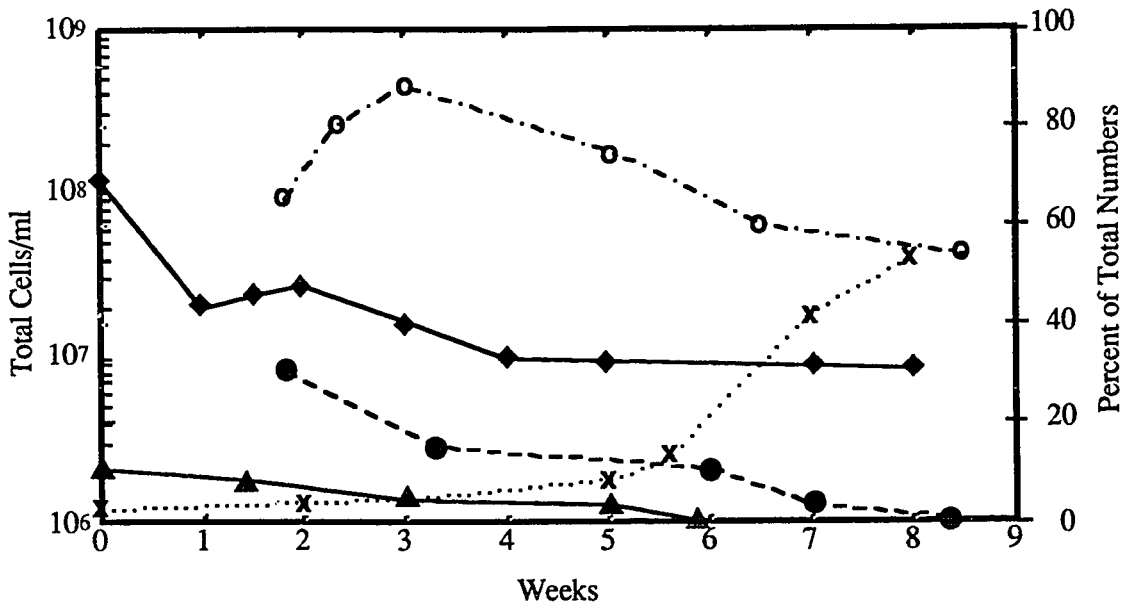


FIG. 3. The total concentration of cells ( ◆, Total cells per mL ), and proportion of four morphological cell types (percent of total cells: o, typical spheroplasts; ●, partial spheroplasts; ▲, residual spirochetes and x, atypical spheroplasts) maintained in modified BSK II at 34°C for nine weeks. (Observations for percent of total numbers was not made on week 0 or 1 because clumping made their quantitation difficult).

FIG. 4. Fluorogram of newly synthesized [$^{35}$S] methionine labeled proteins from whole cell lysates of *B. burgdorferi* and spheroplasts. Spirochetes were grown at 34°C prior to conversion to spheroplasts, and spirochetes and spheroplasts were subjected to a 4-hr labeling period at either 30° or 38°C. Lanes: M, Molecular mass standards; A, *B. burgdorferi* 30°C; B, *B. burgdorferi* 38°C; C, Spheroplast 30°C; D, Spheroplast 38°C. Molecular mass standards as follows: Phosphorylase b (97.4 kDa), bovine serum albumin (66.2 kDa), ovalbumin (42.7 kDa), carbonic anhydrase (31 kDa), and soybean trypsin inhibitor (21.5 kDa).

FIG. 5. Coomassie blue stained proteins of whole cell lysates of *B. burgdorferi* and spheroplasts. Lanes: M, Molecular mass standards; A, *B. burgdorferi* 30°C; B, *B. burgdorferi* 38°C; C, Spheroplast 30°C; D, Spheroplast 38°C. Incubation conditions and molecular mass markers as described in the legend to Fig. 4.

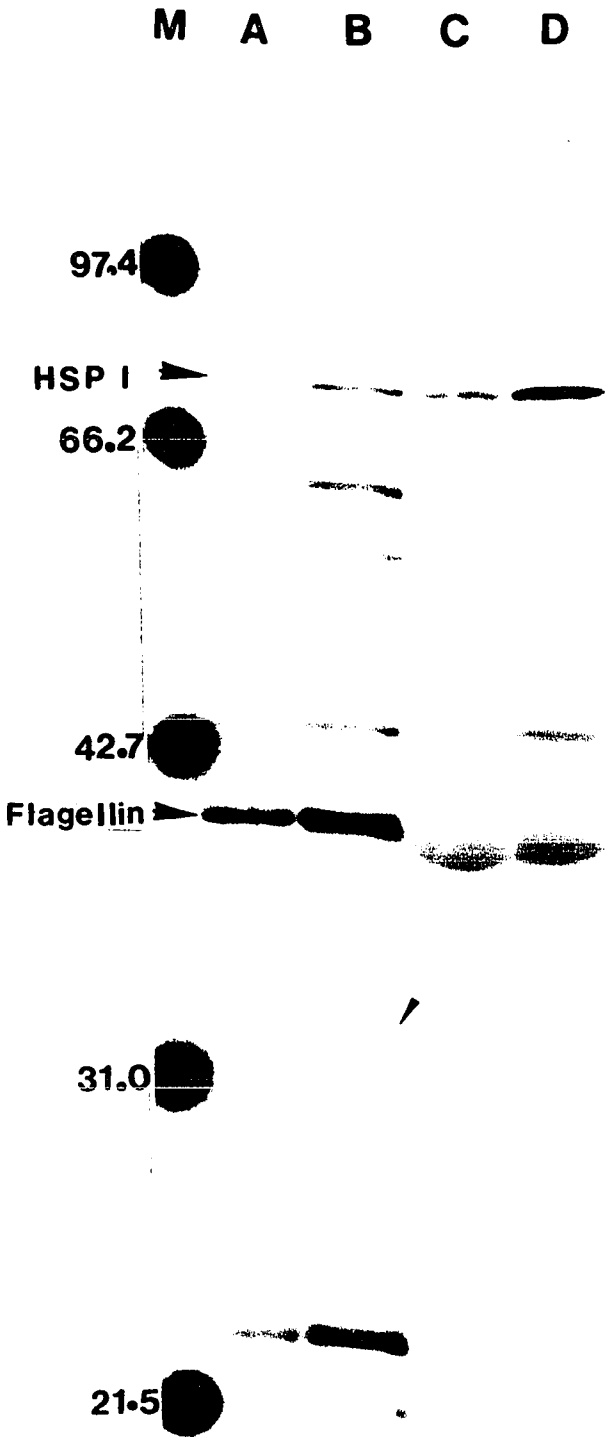


FIG. 4

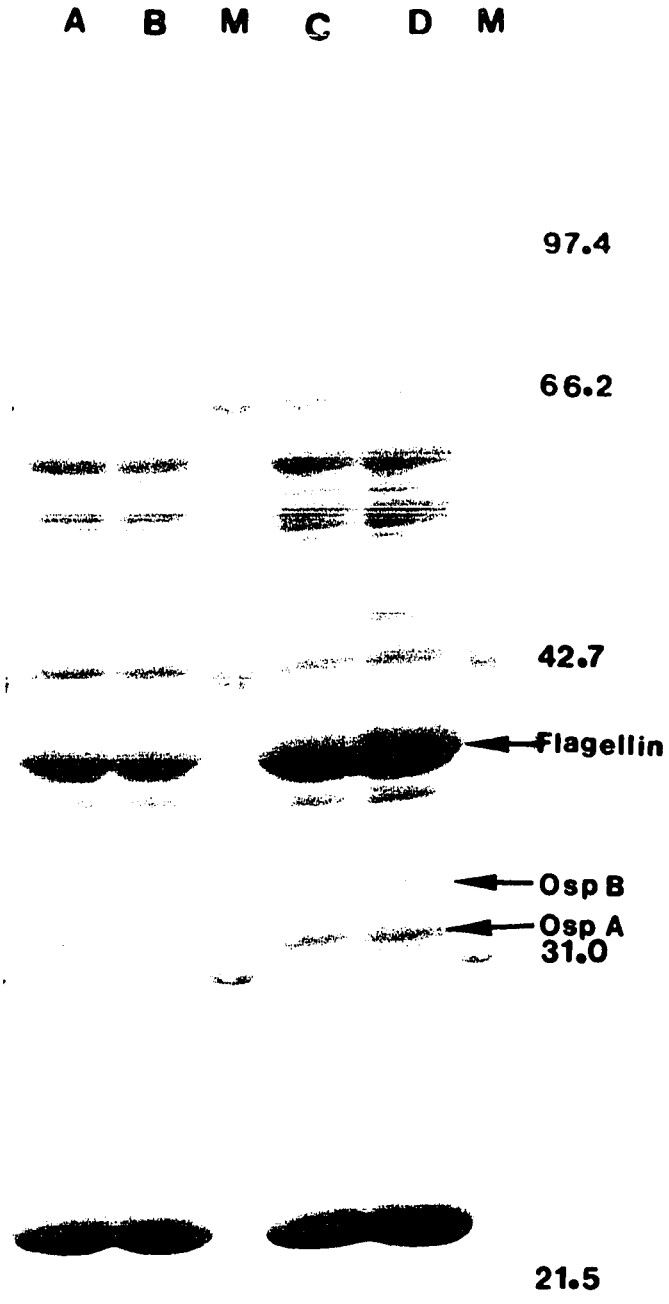


FIG. 5

FIG. 6. Section of *B. burgdorferi* spheroplast. Extended outer membrane surrounds cytoplasmic membrane. Peptidoglycan layer not apparent. Ghost of spirochete in longitudinal section in center of frame. Numerous membrane-bound vesicles (arrows). Magnification, x75,000. Bar = 0.20μm.

FIG. 7. Section of intact *B. burgdorferi.* Cell wall is indistinguishable from cytoplasmic membrane and is surrounded by outer membrane. Magnification, x63,000. Bar = 0.40μm.


FIG. 8. Transmission electron micrograph of *B. burgdorferi*. (A) Negative stain of intact spirochete. Magnification, x15,600. Bar = 1.0μm. (B) Expanded area showing flagella beginning to pull away from cell. Magnification, x32,200. Bar = 0.33μm.


FIG. 9. Transmission electron micrograph of *B. burgdorferi* following Tris Buffer wash. (A) Cell has lost helical configuration. Numerous flagella are visible. Magnification x15,600. Bar = 1.0μm. (B) Expanded area showing cell boundary disruption. Magnification x38,000. Bar = 0.5μm.

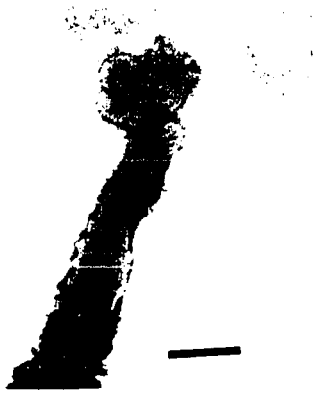

FIG. 10. Transmission electron micrograph of *B. burgdorferi* showing outer membrane "blebs" following phosphate buffered saline and Tris buffer washes. Magnification x42,000. Bar = 0.25μm.


FIG. 11. Transmission electron micrograph of *B. burgdorferi* following Tris buffer wash and lysozyme incubation. (A) Helical coils have disappeared. Numerous, long flagella extend from the spirochete. Magnification x25,000. Bar = 1.0μm. (B) Boundary of cell is beginning to break down allowing underlying membrane to extrude. Magnification x 34,000. Bar = 0.5μm.

FIG. 12. Transmission electron micrograph of *B. burgdorferi* spheroplast. (A) The distended cytoplasmic membrane is beginning to burst (arrows). Magnification, x50,000. Bar = 0.20μm. (B) Spheroplast with bundles of flagella. Magnification x24,000. Bar = 1.0μm.


Fig 12

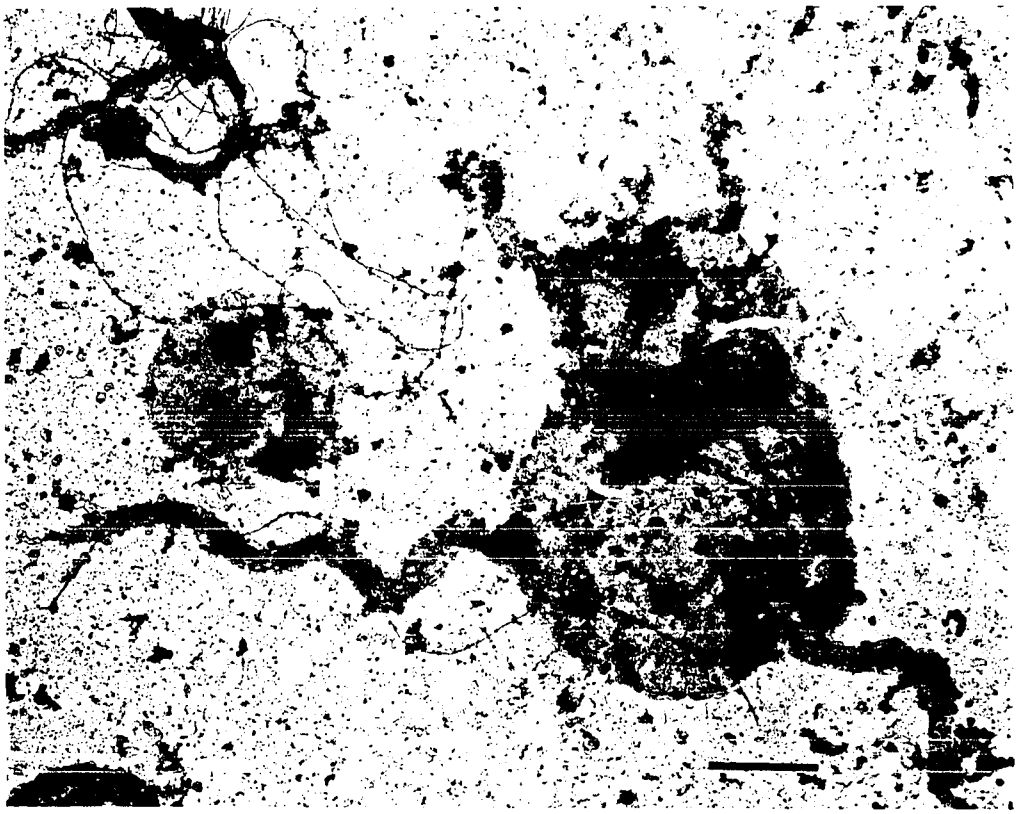

FIG. 13. Transmission electron micrograph of a *B. burgdorferi* partial spheroplast. The distended membrane area is midway along the spirochete length. Magnification x15,000. Bar = 1.0 μm.

FIG. 14. Coomassie blue stained protein of spheroplast lysates after mechanical disruption and centrifugation. Flagellin appears in both fractions. Lanes: M, Molecular mass markers; A, Precipitate; B, supernatent. Molecular mass standards as follows: Phosphorylase b (97.4 kDa), bovine serum albumin (66.2 kDa), ovalbumin (42.7 kDa), carbonic anhydrase (31 kDa), soybean trypsin inhibitor (21.5 kDa) and lysozyme (14.4 kDa).

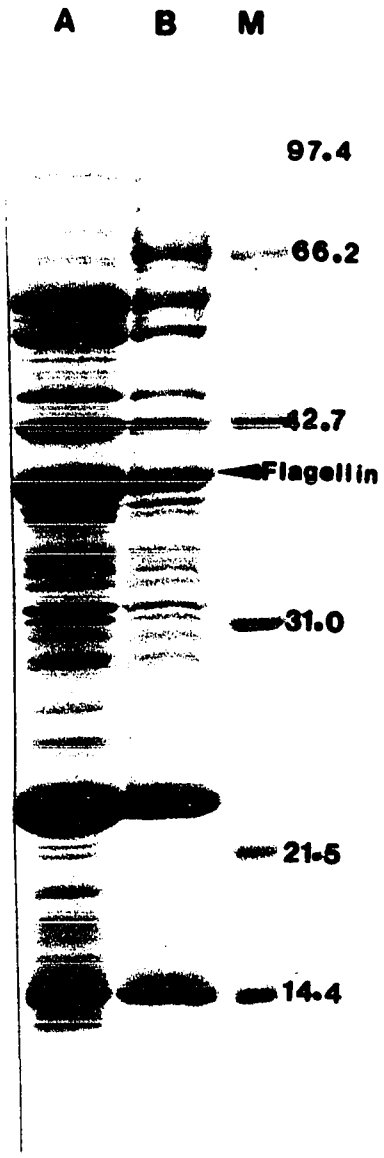


Fig 14